RGD-Peptide Functionalized Graphene Biomimetic Live-Cell Sensor for Real-Time Detection of Nitric Oxide Molecules

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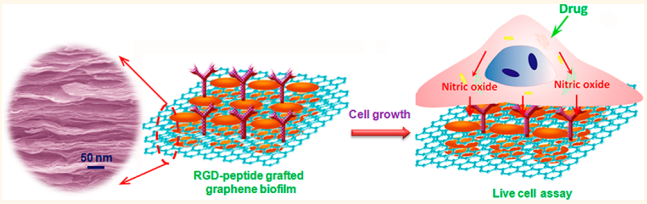
ABSTRACT



It is always challenging to construct a smart functional nanostructure with specific physicochemical properties to real time detect biointeresting molecules released from live-cells. We report here a new approach to build a free-standing biomimetic sensor by covalently bonding RGD-peptide on the surface of pyrenebutyric acid functionalized graphene film. The resulted graphene biofilm sensor comprises a well-packed layered nanostructure, in which the RGD-peptide component provides desired biomimetic properties for superior human cell attachment and growth on the film surface to allow real-time detection of nitric oxide, an important signal yet short-life molecule released from the attached human endothelial cells under drug stimulations. The film sensor exhibits good flexibility and stability by retaining its original response after 45 bending/relaxing cycles and high reproducibility from its almost unchanged current responses after 15 repeated measurements, while possessing high sensitivity, good selectivity against interferences often existing in biological systems, and demonstrating real time quantitative detection capability toward nitric oxide molecule released from living cells. This study not only demonstrates a facial approach to fabricate a smart nanostructured graphene-based functional biofilm, but also provides a powerful and reliable platform to the real-time study of biointeresting molecules released from living cells, thus rendering potential broad applications in neuroscience, screening drug therapy effect, and live-cell assays.

KEYWORDS: RGD-peptide · graphene · functional biofilm · live-cell assay · nitric oxide detection

Real-time detection of biointeresting molecules released from living cells has fundamental significance in the understanding of cellular functions and pathology, and practical importance in the development of applications in diseases diagnosis and drug discovery.[1−6] Among various biointeresting molecules released from living cells, nitric oxide is an important biological signaling molecule, which plays an important role in the regulation of cell function of the nervous, vascular and immune systems, such as acting as a neurotransmitter and modulating vasodilation.[7−9] Nitric oxide also is involved in the parthenogenesis of Parkinson's disease and tumor angiogenesis.[10,11] Thus, real-time detection of nitric oxide molecules released from living cells is essential to provide its temporal release profile for understanding its physiological and pathological process while rendering a promising platform for practical applications in clinical diagnosis, biological research and drug discovery. Nevertheless, real-time detection of nitric oxide remains a great difficulty due to its trace level release from cells, and more critically, the rapid reaction of nitric oxide with oxygen and its short half-life.[12,13] Therefore, it is very challenging to construct a smart functional sensing platform to real-time capture and detect the nitric oxide molecules when they are released.

In contrast to nitric oxide detection techniques such as fluorescent probes,[14] X-ray photoelectron spectroscopy[15] and reverse-phase high performance liquid chromatography[16] which are indirect and complex, electrochemical sensing allows direct assessment and possesses superior simplicity.[17,18] In the development of electrochemical nitric oxide sensors, most recent works have been focused on exploring nanomaterials owing to their high surface area-to-volume ratio and promoted efficient electron transfer.[19,20] Functional nanomaterials such as single-walled carbon nanotubes,[21] multiwalled carbon nanotubes,[22,23] and gold nanoparticles[24] have been explored in electrochemical nitric oxide sensors, showing improved performance. As a superior carbon material with single-atom thickness, large contact surface area, high electric conductivity and wide electrochemical window,

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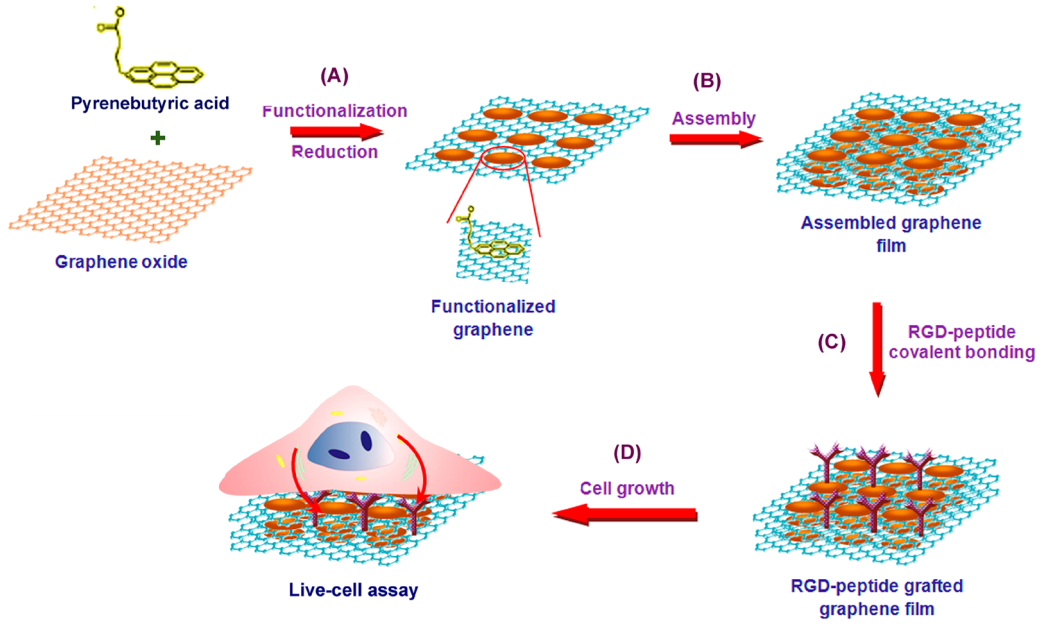

Scheme 1. Schematic illustration of preparation of the free-standing biomimetic film and its live-cell assay. (A) Chemical reduction of graphene oxide and pyrenebutyric acid functionalization; (B) preparation of pyrenebutyric acid functionalized graphene film by filtration of the solution of pyrenebutyric acid functionalized graphene; (C) fabrication of the biomimetic functional film by covalently bonding RGD-peptide on the surface of the pyrenebutyric acid functionalized graphene film *via* EDC/NHS coupling; (D) the functional biofilm as cell adhesion and sensing matrix

graphene has shown various promising applications,[25−33] in a variety of electrochemical sensors.[34−36] Very recently, we have shown that graphene can be used to sensitively detect nitric oxide dissolved in buffer solution.[37] Nevertheless, it is still difficult for graphene to real-time detect nitric oxide molecules released from living cells. The best way is to grow living cells directly on the electrode surface to make them extremely close to the sensor. Thus, an electrode surface should possess excellent biomimetic properties for good cell growth and adhesion, which involves a very complex, highly regulated process and needs specific surface receptors to attach for growth and regulation of cellular functions.[38−40]

Herein we report a novel approach to construct a smart functional biomimetic film sensor by covalently bonding RGD-peptide on graphene surface to significantly boost cell-adhesion and growth for real time electrochemical detection of nitric oxide molecule released from attached human cells under drug stimulations. RGD-peptide is selected because its peptide moiety mimics the cell-binding sequence of extracellular matrix proteins, and it can also protrude to the outer surface of the cellular membrane for promoting good cell adhesive activity.[41,42] The preparation of the functional biofilm is schematically shown in Scheme 1. Graphene oxide is chemically reduced to graphene and at the same time, is functionalized by pyrenebutyric acid that has an aromatic structure to adsorb on graphene surface *via* $\pi-\pi$ interactions. The pyrenebutyric acid functionalized graphene film is then fabricated by a direct assembly approach through filtration of the solution of pyrenebutyric acid functionalized graphene. Then the biomimetic graphene-based film is constructed by covalently bonding RGD-peptide on the surface of pyrenebutyric acid functionalized graphene film *via* ethyl(dimethylaminopropyl) carbodiimide/hydrosulfosuccinimide (EDC/NHS) coupling.

RESULTS AND DISCUSSION

Characterizations of the Graphene-Based Biofilm. Figure 1A shows that the prepared RGD-peptide functionalized graphene biofilm has a free-standing structure, which comprises a well-packed layered structure (Figure 1B) with a thickness of $\sim$8 μm (Figure 1C). The surface of the biofilm has a wave-like morphology, as seen from the top-view scanning electron microscopy (SEM) image in Figure 1D. The surface properties of different graphene-based films were investigated by reflection absorption infrared spectroscopy (RA-IR). Figure 1E shows a strong peak at 1586 cm^{-1} for the pyrenebutyric acid-functionalized graphene film before the covalent bonding of RGD-peptide (curve a), which can be assigned to the stretch mode of a carboxyl group.[32] In contrast, plain graphene without pyrenebutyric acid functionalization displays almost negligible peaks at 1586 cm^{-1} (not shown). These observations suggest the successful functionalization of graphene with pyrenebutyric acid. After covalent bonding of RGD-peptides to the pyrenebutyric acid-functionalized graphene film, the resulted biofilm clearly exhibits two new peaks at 1645 and 1533 cm^{-1}, corresponding to the characteristic amide I and amide II of the

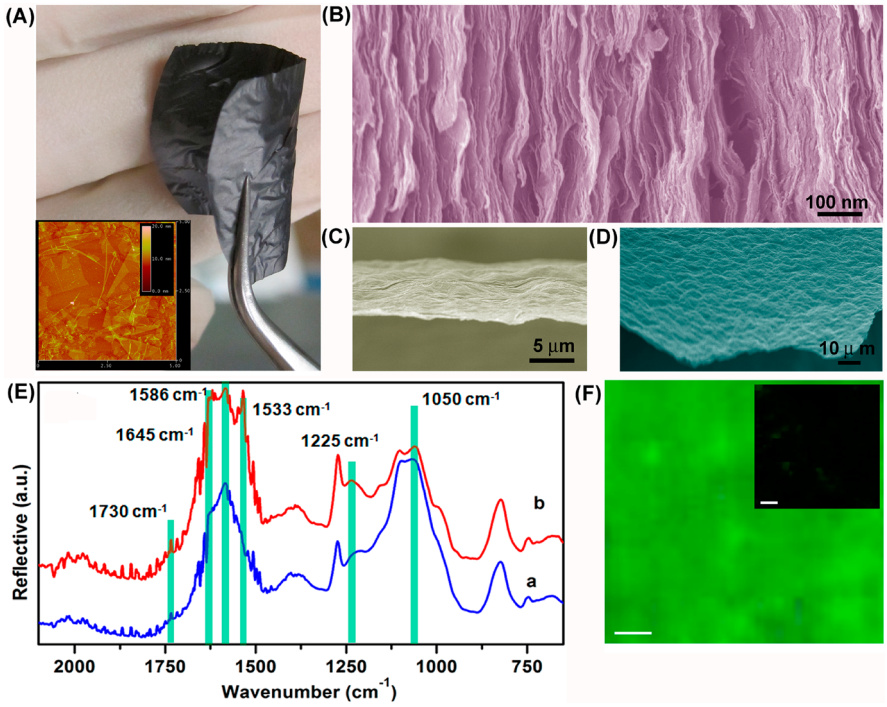

Figure 1. (A) Photograph of RGD-peptide covalently bonded graphene biofilm. The inset of panel A is an AFM image of pyrenebutyric acid functionalized graphene sheets. Side-view (B and C) and top-view (D) SEM images of the RGD-peptide covalently bonded graphene biofilm. (E) RA-IR spectra of (a) pyrenebutyric acid functionalized graphene film and (b) RGD-peptide covalently bonded graphene biofilm. (F) Fluorescence staining of RGD-peptide covalently bonded graphene biofilm and the inset is fluorescence staining of graphene film without RGD-peptide. The scale bars represent 2 mm.

RGD-peptide,[43] whereas the peak at 1586 cm^{-1} assigned to the carboxyl group becomes weak but is still observable (curve b). These results indicate that RGD-peptides are effectively covalently bonded on pyrenebutyric acid-functionalized graphene film and the resulted biofilm still has free carboxyl groups on its surface. The distribution of RGD-peptide on the graphene biofilm is visualized using fluorescein isothiocyanate mixed isomer (FITC) that is highly reactive toward primary amine groups of peptides and proteins. The RGD-peptide functionalized graphene biofilm shows a strong signal and a uniform color distribution, while control experiment of FITC stained graphene film without RGD-peptide conjugation displays a negligible fluorescence signal (Figure 1F), suggesting the uniform distribution of RGD-peptide on the graphene biofilm. It is noted that the graphene biofilm can well keep its intact structure in aqueous solution under mild ultrasonication, exhibiting very good mechanical strength for capability in solution-based manipulations and testings.

Cell Adhesion Behavior on RGD-Peptide Covalently Bonded Graphene Biofilm. To be used for real-time detection of biointeresting molecules released from living cells, the surface property of a sensing matrix is critical because cells need specific surface receptors to attach and grow.[39] As shown in Figure 2A, human umbilical vein endothelial cells cultured on plain graphene film has a stretched shape with pseudopodia, suggesting such a film is a biocompatible platform to promote cell's attachment and growth. It is known that cell adhesion and growth on a substrate are not only dependent on the surface properties of the substrate, but also on the type of proteins that adsorb onto its surface from culture medium and/or secreted from cells,[42] which may contribute to the observed cell adhesion and growth on plain graphene film. The cell attachment and growth ability are significantly improved by the covalent bonding of RGD-peptide, as clearly indicated from the more than 2-fold increase in cell density and nearly 50% increase in cell length over that on plain graphene film (Figure 2C,D). It is understandable that the peptide moiety of the RGD-peptide mimics the cell-binding sequence of extracellular matrix proteins,[41] which provide graphene film biomimetic properties and greatly promotes good cell-adhesive activity and cell growth as well.

Electrochemical Behaviors of RGD-Peptide Covalently Bonded Graphene Biofilm Sensor. The RGD-peptide covalently bonded graphene biofilm was used to detect nitric oxide dissolved in cell culture medium. Figure 3A presents the cyclic voltammetry (CV), of which the biofilm in cell culture medium without nitric oxide (curve 1) exhibits only typical capacitive behavior caused by its double layer over the entire potential range (0.0 to 1.0 V *vs* Ag/AgCl). In contrast, a large oxidation peak with peak potential at 750 mV is observed for CV of the biofilm in cell culture medium

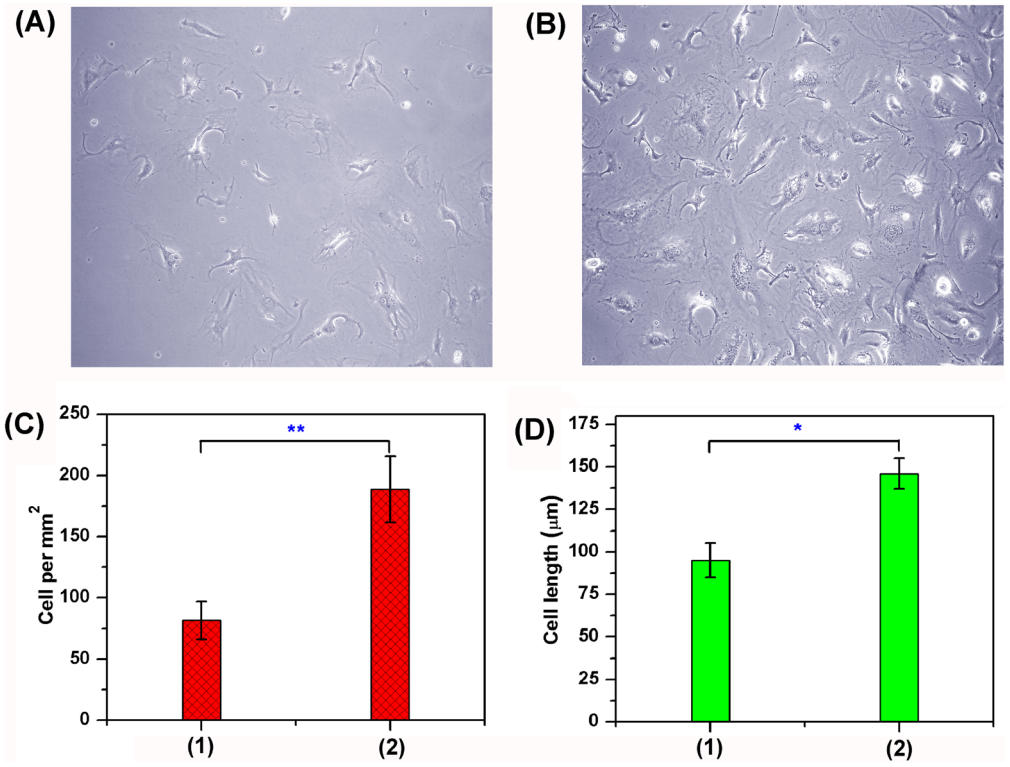

Figure 2. Microscope photographs of human endothelial cells cultured on plain graphene film (A) and RGD-peptide covalently bonded graphene biofilm (B). The number of adhered cells (C) and the cell average length (D) of human endothelial cells cultured on graphene film (1) and RGD-peptide covalently bonded graphene biofilm (2).

containing 10 μM of nitric oxide (curve 2), showing good electrocatalysis toward the oxidation of nitric oxide. During the oxidation, a nitric oxide molecule loses an electron to form NO^+ followed by its chemical converting to NO_2^- as follows:[21,44]

$$NO - e^- \rightarrow NO^+ \quad (1)$$

$$NO^+ + OH^- \rightarrow HNO_2 \rightarrow H^+ + NO_2^- \quad (2)$$

Double potential step chronoamperometry (DPSCA) is a known electrochemical technique to obtain selectable current response with high signal-to-noise (S/N) ratio in a heterogeneous solution. Thus, change of nitric oxide concentration can be monitored by using DPSCA with a double-pulse potential at a regular interval. The typical DPSCA recorded for the dissolved nitric oxide molecules in cell culture medium is shown in the inset of Figure 3B, and the current response is defined as the difference between the first potential (650 mV, onset potential of nitric oxide oxidation) and the second potential (750 mV, the peak potential of nitric oxide oxidation). The logarithmic plot of the current response *versus* the nitric oxide molecule concentration is shown in Figure 3B, showing that there are two linear regions for the biofilm response associated with the increment of the nitric oxide molecule concentration in the range of nanomolar (nM) to micromolar (μM). The detection limit is around 25 nM based on a S/N ratio of 3. With cultured cells the response of the sensing matrix toward nitric oxide is around 1/30 of that of the sensing matrix without cell culture and the detection limit is around 80 nM. The selectivity of the sensing matrix toward the nitric oxide molecule was studied by investigating interfering species such as nitrite, ascorbic acid, and various ions. As displayed in Figure 3C, the RGD-peptide grafted graphene biofilm sensing matrix has a good selectivity toward nitric oxide molecule and can eliminate the interferences from these species including nitrite that has a similar oxidation potential to nitric oxide. The good selectivity of the sensing matrix might come from its retained surface carboxyl groups (as can be seen from Figure 1E) that have negative charge to repel negative charged nitrite and ascorbic acid.

Flexibility and Reproducibility of RGD-Peptide Covalently Bonded Graphene Biofilm Sensor. To examine the flexibility and stability of the GRG-peptide covalently bonded graphene biofilm sensor, its responses by applying bending and relaxing cycles were investigated. As shown in Figure 4, the sensor can almost retain its original current response after 45 bending/relaxing cycles, showing excellent flexibility and stability, which should be contributed from the good mechanical stability of graphene as well as strong integrity of the layered biofilm. The good flexibility of the graphene biofilm should be beneficial for emerging applications in an *in vivo* nitric oxide biosensor to fit the curved measuring surface that is often required.[5]

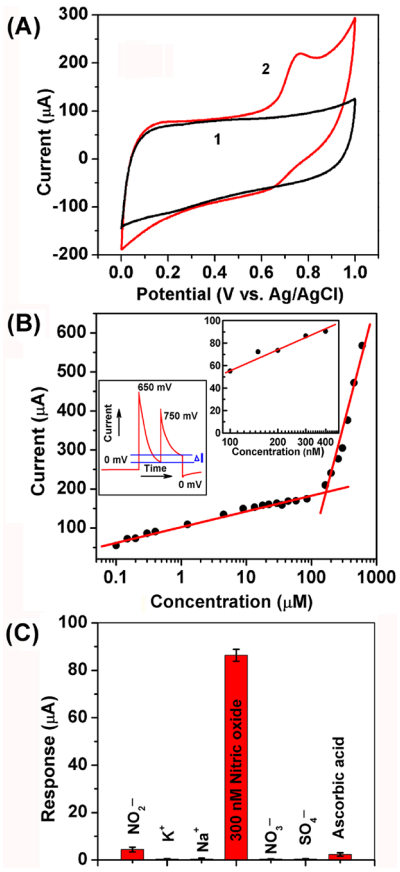

Figure 3. (A) CV curves of the RGD-peptide covalently bonded graphene film in cell culture medium in the absence (1) and presence (2) of 10 μM nitric oxide. Scan rate of 20 mV s^{-1}. (B) The logarithmic plot of the response current *vs* the nitric oxide concentration. Insets of panel B are a typical double potential step chronoamperometry used to obtain the calibration curve and the calibration curve for low concentration nitric oxide. (C) Selective profile of the biofilm.

The response of the sensor is also highly reproducible, as indicated from its almost unchanged current responses after 15 measurements. The deviation of the current responses of over six biofilms prepared with the same procedure is less than 7.6% (relative standard deviation).

Real-Time Detection of Nitric Oxide Molecules Released from Attached Living Cells. During real time molecular detection, a close distance between the nitric oxide-producing cells and the sensing matrix is a critical factor for efficient capture and sensing of nitric oxide molecules that have a limited diffusion distance and are always rapidly metabolized and degraded under cell-culture conditions. Since human endothelial cells are cultured on RGD-peptide covalently bonded graphene biofilm, the close contact should be efficient to real time capture nitric oxide molecule released from living cells for sensitive detection. The biofilm with cultured human endothelial cells was used to demonstrate the real time detecting of nitric oxide molecule released from attached cells. The setup is shown in Figure 5A.

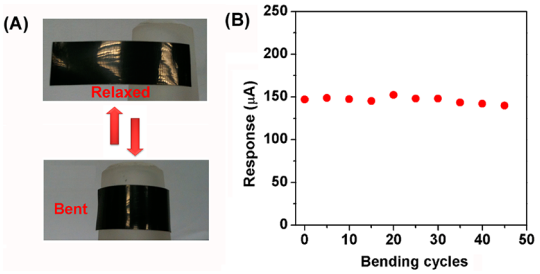

Figure 4. (A) Photographs of the bending and relaxing conditions of the RGD-peptide covalently bonded graphene biofilm. (B) Responses of the biofilm by applying bending.

Acetylcholine (Ach), is selected as a model drug to stimulate cells for nitric oxide release because the signaling pathway of Ach to cell nitric oxide generation has been well understood. It can enable signaling cascades *via* a series of receptors of cells to activate the Ca^{2+}-calmodulin complex and to trigger the generation and release of nitric oxide molecules.[45,46] NG-nitro-L-arginine methyl ester (L-NAME), a specific nitric oxide inhibitor was used as a model drug to inhibit the release of nitric oxide. It is noted that the additions of Ach (1 mM) or L-NAME (1 mM) into the cell culture medium do not cause any significant current change on the graphene biofilm (without cultured cells) during the electrochemical measurements, clearly indicating that both Ach and L-NAME are not electrochemically active.

The dynamic responses of the cells grown on the RGD-peptide covalently bonded graphene biofilm sensor toward different drugs were investigated. The DPSCA responses are shown in Figure 5B. The stimulating and inhibiting agents were added as indicated by the arrow. Both 1 mM and 0.5 mM Ach stimulations caused significant current responses. In contrast, no response is observed for the cells under the stimulation of a mixture of 1 mM Ach and 1 mM L-NAME, which should be attributed to the specific inhibiting behavior of L-NAME toward nitric oxide release. The response under Ach stimulation is a concentration-dependent behavior, and the current response of 1 mM Ach stimulation is more than two times than that of 0.5 mM Ach stimulation after a period of 2 min as shown in Figure 5B,C. Based on the drug stimulated current response and the biofilm sensor performance calibrations, the nitric oxide molecule concentration is around 350 and 145 nM for 1 mM and 0.5 mM Ach stimulation, respectively, demonstrating the real time quantitative nitric oxide detection capability. It has been reported that the concentrations of nitric oxide produced from living bacteria cells are in the range of nanomoles.[47] The concentrations of nitric oxide released from human cells investigated in this work are in a few hundreds of nanomoles (145−350 nM). The reason for the released nitric oxide difference between human and bacterium cells is not fully understood yet,

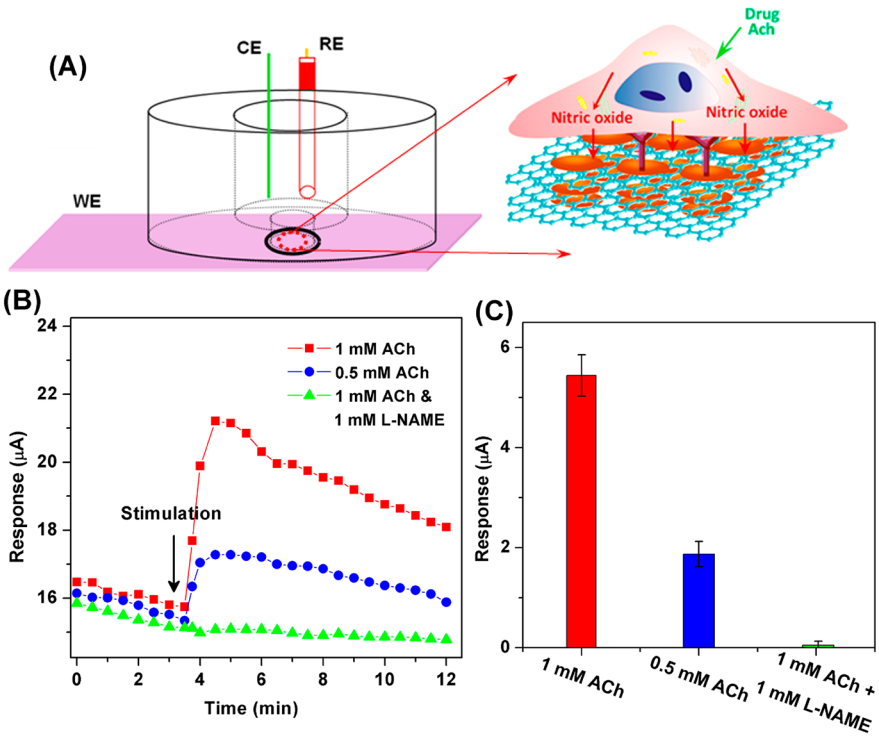

Figure 5. (A) Scheme showing the setup for live-cell assay. (B) Real time monitoring of nitric oxide molecule released from the attached cells on RGD-peptide covalently bonded graphene biofilm in cell culture medium. The drug was added at the time indicated by the arrow. (C) The current responses of the cultured cells on RGD-peptide covalently bonded graphene biofilm toward different drugs. Acetylcholine (Ach) is a model drug to stimulate cell nitric oxide release and NG-nitro-L-arginine methyl ester (L-NAME) is a specific nitric oxide inhibitor.

although we speculate it may be due to the much larger size of the human cell than that of the bacterium one. This is currently under investigation in our lab.

CONCLUSIONS

In brief, we demonstrate a facial approach to construct a smart biomimetic sensor by covalently bonding RGD-peptide on surface of pyrenebutyric acid functionalized graphene film. The RGD-peptide modification makes the film biomimetic for human cell attachment and growth. The biofilm sensor exhibits good flexibility and stability by retaining its original response after 45 bending/relaxing cycles, and high reproducibility as indicated from its almost unchanged current responses after 15 repeated measurements, while showing high sensitivity and good selectivity toward nitric oxide detection. The biofilm sensor was further used to selectively detect the nitric oxide molecule released from attached human endothelial cells under drug stimulations, showing real time quantitative detection capability. This study not only reports an approach to fabricate graphene-based functional biofilm, but also provides a powerful platform to build next generation biomedical devices for live-cell assay and drug therapy effect screening. It is worthy of a note that the effect of electrical signal during measurements on nitric oxide release from cells directly grown on the electrode needs to be further studied. In addition, the cell could continue to grow on the graphene biofilm, and it may provide good knowledge to simultaneously monitor the time-dependent cell density while detecting nitric oxide. We have planned to conduct these experiments.

EXPERIMENTAL SECTION

Pyrenebutyric Acid-Functionalized Graphene. Natural graphite (Fluka) was put into a mixture solution of H_2SO_4, $K_2S_2O_8$, and P_2O_5 with weight ratio of 5:1:1, and the mixture was heated at 80 °C for 6 h. After being stirred overnight at room temperature, the mixture was diluted with deionized water and filtered. The filtered powder was added into H_2SO_4 (ice-cooling) and stirred for 15 min. Then, $KMnO_4$ was added slowly to the mixture with ice-cooling under stirring. The mixture was heated to 35 °C and kept for 6 h, and then slowly diluted with deionized water within an ice-cooling environment. After 2 h at room temperature, hydrogen peroxide (30) was added slowly and stirred for 30 min. It was diluted with deionized water, and centrifuged, washing with 10% aqueous HCl solution a couple of times to obtain graphite oxide. Exfoliation was carried out by sonicating 0.1 mg mL^{-1} graphite oxide aqueous solution under ambient condition for 60 min. To prepare pyrenebutyric acid-functionalized graphene dispersion, 100 mg of NaOH was added to 100 mL (0.1 mg mL^{-1}) of graphene oxide dispersion, and then 150 mg of pyrenebutyric acid was added. The mixture was reduced with hydrazine monohydrate (250 μL) at 80 °C overnight. The dispersion was centrifuged to yield a black supernatant.

RGD-Peptide Covalently Bonded Graphene Biofilm. The pyrenebutyric acid-functionalized graphene dispersion was filtered through a cellulose acetate membrane (pore size of 0.22 μm) to produce a graphene film. The film was repeatedly washed with deionized water and dried under vacuum. Then, the film was immersed in a solution of 4 mM of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and 10 mM of *N*-hydrosulfosuccinimide sodium salt (NHS) for 1 h. After the activated film was gently rinsed with deionized, 1 mg mL^{-1} of RGD-peptide was dropped on its surface and incubated for 2 h to yield RGD-peptide covalently bonded graphene biofilm, followed by a rinse with pH 7.4 phosphate buffer.

Cell Culture and Manipulation. Culture medium was prepared by mixing MCDB 131 medium, sodium bicarbonate, heparin, bovine bone extract, and fetal bovine serum in autoclaved deionized water and filtered. Human umbilical vein endothelial cells (HUVECs) were cultured in as-prepared culture medium at 37 °C in a humidified incubator (95% air with 5% CO_2). To culture cells on graphene films, HUVECs were seeded at density of ~5000 cell cm^2 and allowed to stay at 37 °C in a humidified incubator (95% air with 5% CO_2) for different times. Cell density grown on graphene biofilm was found to be 0, 132, 189, 225, and 241 per mm^2 at time 0, 12, 18, 30, and 42 h, respectively. It can be found that cells growth rate became slow after 18 h, which was chosen as the time to culture cells for cell sensing study.

Apparatus and Characterizations. Pyrenebutyric acid-functionalized graphene sheets were characterized by atomic force microscopy (AFM, Nanoman, Veeco, Santa Barbara, CA) using tapping mode. The morphologies of biofilms were investigated by scanning electron microscopy (SEM, JSM-6700F, Japan). Fluorescent images for fluorescein isothiocyanate mixed isomer (FITC) labeled graphene films were acquired with a proteomic imaging system (Perkin-Elmer) with an excitation wavelength of 494 nm and emission wavelength of 518 nm. Phase-contrast images of cells cultured on graphene films were taken with an inverted microscope (Olympus IX71). Electrochemical measurements were performed in a three-electrode system using CHI-760D electrochemical station (CH Instruments Inc. USA) with the biofilm (with or without cultured cells), Pt wire and Ag/AgCl as working, counter, and reference electrodes, respectively. For the interfering study, 300 nM nitric oxide was used while keeping all other interfering species at the concentration of 25 μM.

Real Time Monitoring Cell Released Nitric Oxide Molecules. The setup of the device for real time monitoring cell released nitric oxide molecules is the same as that of electrochemical measurement. The diameter of the low columniform is 2 mm and that of the higher one is 20 mm. A 2 mL cell culture medium was filled as the electrolyte. Cell nitric oxide production behavior was monitored by using double potential step chronoamperometry. During cell released nitric oxide measurement, the cell culture medium inside the device was mildly stirred such that measured nitric oxide concentrations can be considered to be the average concentrations.

Conflict of Interest: The authors declare no competing financial interest.

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